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PRINCIPAL INVESTIGATOR: Honglin Li, Ph.D.

CONTRACTING ORGANIZATION: Children's Memorial Hospital  
Chicago, Illinois 60614

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## INTRODUCTION:

Apoptosis or programmed cell death is a cell intrinsic suicidal mechanism, which is essential for tissue homeostasis and animal development. Dysregulation of this process may lead to a number of human diseases, including prostate cancer (PC). Resistance of PC cells to apoptosis plays important roles in the pathogenesis and progression of prostate cancer. Some of androgen-independent cancer cells that do not undergo apoptosis after androgen ablation become apoptosis-resistant and metastatic, frequently due to upregulation of anti-apoptotic Bcl-2 and loss-of-function of p53 (Bookstein et al, 1993; Eastham et al, 1995; Cardillo et al, 1997). As a result, metastatic prostatic cancer, usually resistant to conventional anti-tumor therapies, is a lethal disease without curative therapy. Therefore, an understanding of apoptotic machinery and its regulatory mechanism in PC cells is critical for us to develop an effective therapy to combat metastatic prostate cancer. The TNF-related apoptosis-inducing ligand, TRAIL (also called Apo2L), has recently been emerging as a non-toxic anti-cancer agent because it is capable of inducing apoptosis in many of tumor cell lines but not in normal cells tested (Wiley et al, 1995; Pitti et al, 1996). In this project, we will examine the effects of TRAIL treatment in androgen-dependent and -independent PC cells, to dissect the TRAIL-induced signaling pathway in those cell lines, and to characterize the synergistic effects of TRAIL with other therapeutic agents, and finally to test the anti-tumor effect of TRAIL in the animal models of prostate cancer. Through these works, we will be able to firmly establish the foundation for a novel therapy of prostate cancer.

## BODY:

*Task 1:* To examine the TRAIL-induced apoptotic pathway in prostate cancer cells (months 1-12):

- To survey TRAIL sensitivity of PC cancer cells (months 1-6)
- To examine the effect of cycloheximide on TRAIL sensitivity of prostate cancer cells (months 6-9)

Please see the report of year 2002.

- To identify the possible molecular determinants of TRAIL sensitivity (month 6-12)

We have continued to work on this project and combined previous and current results together.

## Results:

To identify the possible molecular determinants of TRAIL sensitivity, we have screened the known regulators for TRAIL signaling pathway.

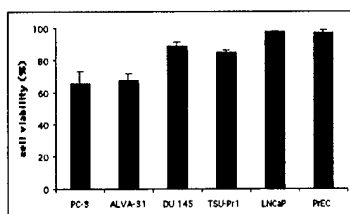
### 1) Effect of TRAIL on human prostate normal and cancer cells

Recombinant human TRAIL (residues 95-281) was produced in *Escherichia coli* as a fusion protein containing an N-terminal His-tag, and purified by using standard nickel affinity chromatography. Cytotoxic effects of TRAIL on human prostate normal epithelial cells (PrEC) and cancer cell lines (namely, PC-3, TSU-Pr1, DU 145, ALVA-31 and LNCaP) were evaluated by MTT assay. As shown in Figure 1A, PC-3 and ALVA-31 cells were sensitive to TRAIL, and DU 145 and TSU-Pr1 revealed moderate sensitivity, while LNCaP and normal PrEC cells were fully resistant to TRAIL treatment.

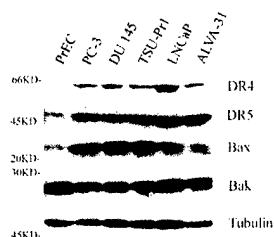
### 2) Expression of TRAIL receptors and pro-apoptotic proteins in prostate cancer cells

As shown in Figure 1, no correlation was found between the TRAIL sensitivity and the expression of TRAIL receptors, and Bax/Bak.

A.



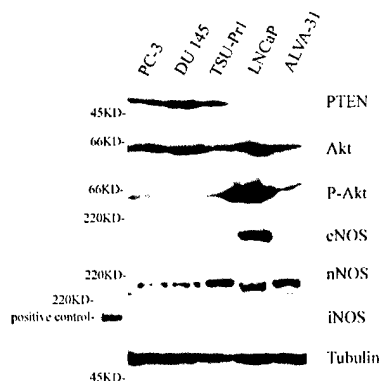
B.



**Figure 1.** Effect of TRAIL on cell viability of human prostate normal and cancer cells. A. The effect of TRAIL on cell viability. Cells were treated with TRAIL (500ng/ml) for 24 h, and viability was measured by MTT assay. Data represent mean  $\pm$  S.E. B. Expression of TRAIL receptors and pro-apoptotic proteins in prostate cancer and normal cells. Cell lysates prepared from prostate normal and cancer cells were probed by immunoblotting with anti-DR4, anti-DR5, anti-Bax, and anti-Bak antibodies.

## 2) LNCaP Cells expresses high levels of active Akt and eNOS protein

Previous studies have demonstrated that LNCaP cells have a high level of constitutive active Akt due to the deletion and mutation at PTEN alleles (Thakkar et al, 2001). Inhibition of PI3K activity using wortmannin and LY-294002 suppresses constitutive Akt activity and sensitizes LNCaP cells to TRAIL. Conversely, overexpression of constitutively active Akt attenuates TRAIL-induced apoptosis (Nesterov et al, 2001; Thakkar et al, 2001). By repeating these experiments, we found that there was no PTEN expression in LNCaP cells, in which the highest Akt activity was observed, although total Akt protein levels among these prostate cancer cells were similar (Fig. 2). Another interesting substrate is eNOS, whose activity is enhanced when modified by Akt (Dimmeler et al, 1999; Fulton et al, 1999). To explore whether eNOS may be involved in TRAIL-resistance, we examined the expression of three isoforms of NOS among the five prostate cancer cells. As shown in Fig. 2, only LNCaP cells expressed eNOS, whereas all of them expressed nNOS, but do not express iNOS.



**Figure 2.** LNCaP cells express high level of eNOS. Cell lysates from five prostate cancer cell lines were probed by immunoblotting with anti-PTEN, anti-Akt, anti-phospho-Akt, anti-nNOS, anti-iNOS and anti-eNOS.

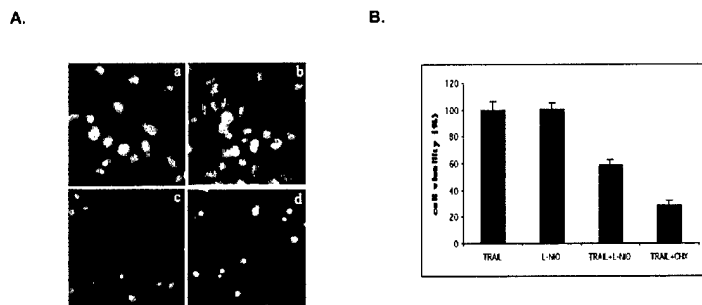
## 3) NOS inhibitor sensitized LNCaP cells to TRAIL-induced apoptosis

To further examine whether the high-level expression of eNOS in LNCaP cells contributes to TRAIL resistance, we used eNOS inhibitor to test if inhibition of eNOS can sensitize LNCaP cells to TRAIL. L-NIO is a potent inhibitor of eNOS compared to other arginine analogs (Rees et al, 1990). LNCaP cells were pre-treated with L-NIO for 2 h before exposure to TRAIL (500ng/ml), and cell death was evaluated by nuclear morphology and measured by MTT assay after 24 h treatment. As shown in Fig. 3A and 3B, TRAIL or L-NIO alone did not induce any cell death (2% cell death), but pretreatment with L-NIO or protein synthesis inhibitor (cycloheximide) sensitized LNCaP cells to TRAIL-induced apoptosis (38% and 76% cell death, respectively).

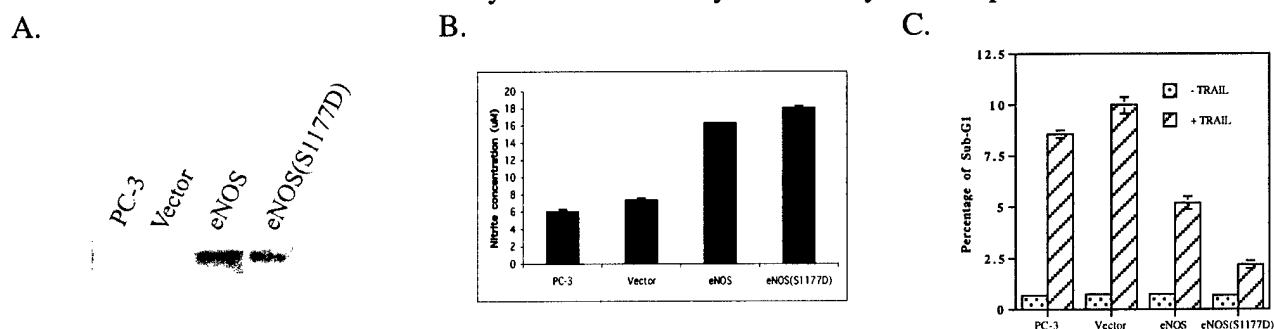
## 4) eNOS expression partially inhibits TRAIL-induced apoptosis of PC-3 cells

To further test this possibility, we used PC-3 cells as a model system to test the effect of eNOS on TRAIL-induced apoptosis, because PC-3 cells were most sensitive to TRAIL among the five prostate cancer cells we tested (Fig. 1A) and have no expression of eNOS (Fig. 2). Wild type eNOS and mutant eNOS (S1177D) (Mutation of serine 1177 into aspartic acid that substitutes for the negative charge afforded by the addition of phosphate and mimics the activation state induced by Akt) were introduced into PC-3 cells by gene transfection, and stable clones were selected. We randomly selected 30 G418-resistant clones, among which 6 clones expressed wild-type eNOS and 4 clones expressed eNOS (S1177D), respectively (Fig. 4A). The clones with high-level expression of eNOS were tested for their

TRAIL sensitivity. Cells were treated with TRAIL (500ng/ml) for up to 24 h, and the DNA content was analyzed by flow cytometry. The sub-G<sub>1</sub> DNA peak, characteristic of apoptotic cell death, was clearly detectable in PC-3 cells (8.6%±0.3%) or empty vector clones (10.0%±0.7%) treated with TRAIL with respect to untreated controls, while eNOS-expressing cells have much lower sub-G<sub>1</sub> peak (5.2%±0.3%), and the lowest sub-G<sub>1</sub> peak was observed in eNOS (S1177D)-expressing cells (2.4%±0.1%) (Fig. 4C). These results demonstrated that overexpression of eNOS in TRAIL sensitive PC3 cells can inhibit TRAIL-induced apoptosis, further indicating the role of eNOS in regulation of TRAIL signaling.



**Figure 3.** Inhibitor of eNOS sensitizes LNCaP cells to TRAIL. A. DNA staining of LNCaP cells treated with 500ng/ml TRAIL alone (a), 20mM L-NIO alone (b), TRAIL and L-NIO combination (c) or TRAIL and 10μg/ml cycloheximide combination (d). The cells were stained with Hoechst 33342 (5 μg/ml) and nuclear morphology was observed by fluorescent microscope. B. MTT assay of cell viability. Cell viability of LNCaP cells treated for 24 h with 500ng/ml TRAIL, 20mM L-NIO, 10μg/ml cycloheximide alone or in combinations. The viability was measured by MTT assay. Data represent means ± S.E.



**Figure 4.** Overexpression of eNOS in PC-3 cells partially inhibit TRAIL-induced apoptosis. A. eNOS or eNOS (S1177D)-expressing clones. G418-resistant clones were expanded and cell lysates were probed with anti-eNOS antibody. B. eNOS activity in eNOS stable cell lines. Culture media from control PC-3 cells, empty vector clones, eNOS and eNOS (S1177D)-expressing clones were collected, and nitrites release was measured by using NO Assay kit (Molecular Probes Inc.). C. The percentage of cell death evaluated by flowcytometry. Cells ( $1 \times 10^6$ ) were treated with or without TRAIL (500ng/ml) for 24 h, stained with propidium iodide, and DNA content was analyzed by flowcytometry. The percentage of sub-G1 represented the percentage of cell death.

**Task 2:** To dissect the signaling pathway of TRAIL-induced apoptosis in PC cells (months 12-24):

- The role of a caspase cascade in TRAIL-induced apoptosis (months 12-15)
- The role of DISC formation in TRAIL-induced apoptosis (months 15-24)
- The role of mitochondria in TRAIL-induced apoptosis (months 15-24)

## Results:

### 1) Both caspase cascade and mitochondrial pathway are important for TRAIL-induced apoptosis

To further dissect the signaling pathway of TRAIL-induced apoptosis in PC cells, we transiently

expressed various inhibitors in PC-3 cells and examined the TRAIL sensitivity. As shown in Figure 5, pan-caspase inhibitor zVAD-fmk, caspase-8 dominant negative mutant and FADD dominant negative mutant can effectively inhibit TRAIL-induced apoptosis of PC-3 cells, indicating that caspase cascade and DISC formation are important for initiation of TRAIL signaling. To evaluate the mitochondria, we established stable cell lines to overexpress Bcl-xL, a potent inhibitor of apoptosis. As shown in Figure 6, overexpression of Bcl-xL can inhibit TRAIL-induced apoptosis in the absence and presence of cycloheximide (only the presence of cycloheximide is shown here). This result suggests that both caspase cascade and mitochondrial pathway contribute to TRAIL-induced apoptosis.

Figure 5

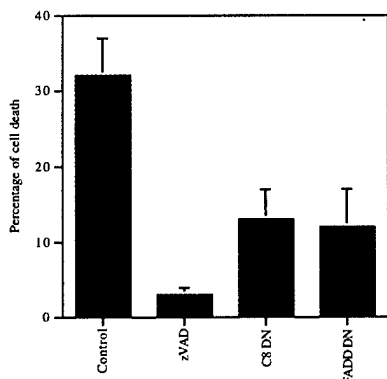
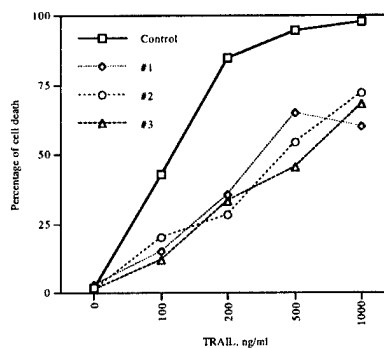


Figure 6



**Figure 5:** TRAIL-induced apoptosis of PC-3 cells is caspase-dependent. PC-3 cells were co-transfected with various constructs along with EGFP. After 24-hr transfection, cells were treated with TRAIL (500 ng/ml) for additional 24 hours. Percentage of cell death was scored by counting dead and alive green fluorescent cells.

**Figure 6:** Overexpression of Bcl-xL partially inhibits TRAIL-induced apoptosis of PC-3 cells. Three stable lines expressing Bcl-xL and control cell lines were treated with various amounts of TRAIL in the presence of cycloheximide (10  $\mu$ m/ml) for 24 hours. Percentage of cell death was evaluated by MTT assay.

## 2) Initial characterization of TRAIL receptors-interacting proteins:

It is generally believed that TRAIL signaling is similar to Fas signaling pathway, in which cross-linking of death receptors leads to the formation of Death-Inducing-Signaling-Complex (DISC) and activation of caspase-8. Active caspase-8 activates downstream caspase cascade and induces mitochondrial damage via BID cleavage, and results in apoptotic cell death. Nonetheless, TRAIL signaling is still poorly understood. To further understand TRAIL signaling in PC cells, we did a yeast 2-hybrid screening to identify proteins interacting with TRAIL receptors. We used the intracellular domains of TRAIL receptor DR4 and DR5 as baits and Clontech human prostate cDNA library. In order confirm the specificity, we used the DED domain of caspase-10 and the CARD domain of caspase-2 as negative controls. The initial identification of specific clones was summarized in Table 1. Although more work is needed to confirm the finding, there are a few interesting points that we can make by initial identification of these clones:

- Interestingly, not many clones can interact both DR4 and 5 except Filamins even though DR4 and 5 share high sequence homology. We currently do not have a good explanation for this observation.
- Multiple clones, namely UBC9 Saltzman et al, 1998) , Daxx (Yang et al, 1997) , Flash (Imai et al, 1999) and SODD (Jiang et al, 1999), have been demonstrated to interact with other death receptors such as Fas and TNFR1, indicating that DR5 may share a similar signaling pathway to Fas/TNFR1. This may also indirectly vindicate our screening result.
- Both UBC9 and PIASs are the components of sumoylation machinery (Jackson, 2001) Previous studies suggested the possible role of Sumo/Sentrin in regulation Fas-induced apoptosis (Okura et al, 1996). Further study will be needed to examine the role of sumoylation in TRAIL signaling.

**Table1:** Potential TRAIL receptors-interacting proteins identified by yeast 2-hybrid screening:

DR4-interacting clones		DR5-interacting clones	
Clone Names	Hits	Clone Names	Hits
DNAJB1	6	UBC9	130
DNAJB2	1	Daxx	1
DNAJB4	2	CGI99	14
DNAJB6	3	Filamin A	3
FKBP8	1	APOE	2
BAT3	1	Metallothionein	2
Calponin 3	1	RAP80 (zinc finger protein)	6
Filamin A	10	G protein-coupled receptor kinase 6	1
Filamin B	2	FLASH	14
CAP	1	PIAS1	2
SHARP (SMART/HDAC1 associated protein)	1	PIASx	3
LAF4 (lymphoid nuclear protein)	1	SODD	4
Ribosomal protein S20	2	Coatmer protein complex subunit 2	2
Fatty acid binding protein 4	2	Poly (rC)-binding protein 1	2
Integrin beta 4	1	Echinoderm microtubule associated protein 2	2
C-terminal binding protein 1	2	Lethal giant larvae homolog 2	1
HNRP H1	1	Fibromodulin	1
Steroid receptor coactivator-1	1	SECIS binding protein 2	1
Similar to DKFZP434H132 protein	2	Clone B271E1	1
KIAA1064	2	Clone RP11-571I18	1
KIAA0171	1	Clone RP11-522I20	1
CG8055	1	Clone RP11-33I11	1
FLJ21652	1	Clone RP43-180A19	1
Clone RP11-437B10	1	Clone RPCI11-352M15	1
Clone 4402152	1	IgG heavy chain (G3m)	2
IgG HC	9	Annexin A6	1
Novel	1	Novel	1
		Novel	1

**Task 3:** To characterize the effect of TRAIL and the combined effects of TRAIL and other therapeutic agents on PC in vitro and in vivo (months 12-36):

- a. Examine the possible synergistic effects between TRAIL and conventional therapies in vitro (months 12-36)
  - a) The effects of conventional agents on TRAIL sensitivity of PC cells:
  - b) The effects of conventional agents on TRAIL sensitivity of PC cells overexpressing Bcl-2/Bcl-xL
- b. Examine the possible synergistic effects between TRAIL and conventional therapies in vivo (months 12-36)
  - a) Orthotopic model of human PC cell lines (LNCaP and PC-3 cells)
  - b) Transgenic Adenocarcinoma of Mouse Prostate Model (TRAMP)



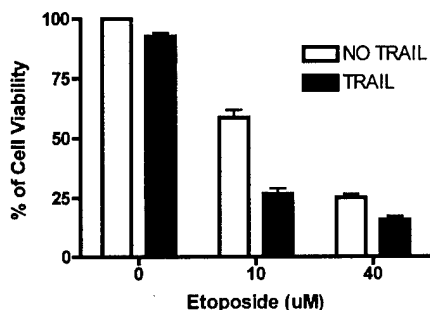
## Results:

### 1) Synergistic effect with the combination of TRAIL and conventional chemotherapies

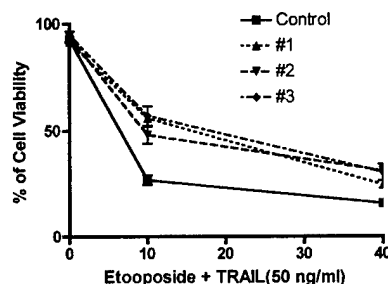
#### a. DNA topoisomerase II inhibitor etoposide sensitized PC3 cells to TRAIL

Etoposide is a DNA topoisomerase II inhibitor commonly used as chemotherapeutic agent. As shown in Figure 7A, treatment of anti-cancer therapeutic agent etoposide greatly enhanced the sensitivity of PC3 cells to low concentration of TRAIL (50 ng/ml). Additionally, Bcl-x<sub>L</sub> partially inhibited apoptosis induced by TRAIL and etoposide, indicating the important role of mitochondrial pathway.

A.



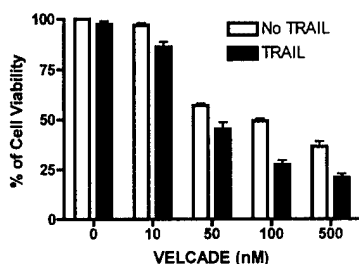
B



**Figure 7:** Anti-cancer agent etoposide sensitized PC3 cells to TRAIL-induced apoptosis. A. PC3 cells were treated with TRAIL (50 ng/ml) and etoposide as indicated for 24 hours, and the cell viability was determined by CellTiter Blue assay (Promega). B. PC3/Bcl-x<sub>L</sub> stable cell lines were treated with TRAIL and etoposide, and the cell viability was scored as described in A.

#### b. Proteasome inhibitor VELCADE sensitized PC3 cells to TRAIL

VELCADE (Millennium) is a potent proteasome inhibitor that has been used for treatment of multiple myeloma. As shown in Figure 8, low concentration of VELCADE sensitized PC3 cells to TRAIL (50 ng/ml). Similar effect was observed for another proteasome inhibitor MG132 (data not shown).

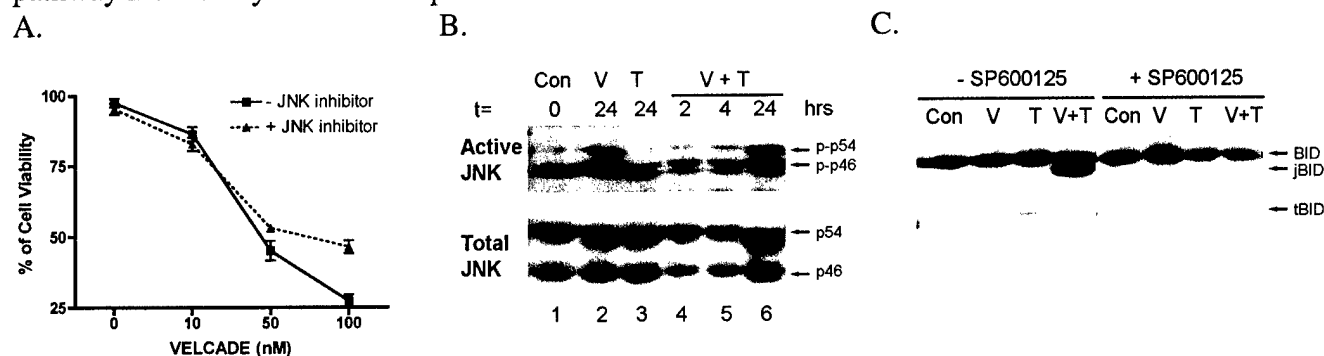


**Figure 8** Proteasome inhibitor VELCADE sensitized PC3 cells to TRAIL-mediated apoptosis. PC3 cells were treated with TRAIL (50 ng/ml) and various amounts of VELCADE as indicated for 24 hours, and cell viability was scored using CBT assay.

#### c. JNK inhibitor partially inhibited the synergistic effect of TRAIL and VELCADE

JNKs (also known as stress-activated protein kinases) is a subgroup of the MAPK superfamily that is activated by cells stresses such as UV or cytokines (Varfolomeev and Ashkenazi, 2003). JNK phosphorylates transcriptional factors such as c-JUN, and turns on genes that controls proliferation, differentiation and apoptosis. However, its precise role in apoptosis remains somehow controversial as its effect depends on the type of cell and death stimuli. It was shown that JNK can be activated by inhibition of proteasome (Hideshima et al, 2003). Thus, we investigated the role of JNK in VELCADE's effect on TRAIL sensitivity of PC cells. As shown in Figure 9A, small JNK inhibitor SP600125 partially inhibited apoptosis induced by low concentrations of VELCADE and TRAIL (74% of cell death versus 54%, at 100 nM of VELCADE and 50 ng/ml of TRAIL). This result strongly suggests that JNK activation may be partly responsible for the synergistic effect between VELCADE

and TRAIL. As illustrated in Figure 9B, JNK was activated by VELCADE alone (lane 2) and the combination of VELCADE and TRAIL (lane 6). Recently, JNK-dependent BID cleavage is required for TNF $\alpha$ -induced caspase-8 activation and apoptosis (Deng et al, 2003). We examined if JNK-dependent BID cleavage occurred in our system. Interestingly, BID was cleaved to jBID (JNK-mediated cleaved BID) only when cells were treated with both VELCADE and TRAIL (Figure 9C). Furthermore, JNK inhibitor SP600125 fully inhibited the production of jBID, indicating that conversion of BID to jBID is indeed JNK-independent. The role of JNK and jBID in the apoptotic signaling pathway induced by TRAIL and proteasome inhibitor will be further defined.



**Figure 9.** JNK is partially required for apoptosis induced by TRAIL and VELCADE. A. JNK inhibitor SP600125 partially inhibited apoptosis of PC3 cells induced by VELCADE and TRAIL. Cells were treated with TRAIL (50 ng/ml) and various amounts of VELCADE as indicated for 24 hours, and cell viability was scored by CBT assay. B. JNK is activated by VELCADE. PC3 cells were treated with VELCADE alone, TRAIL alone, and both, and the active JNK was examined by anti-phosphorylated JNK (Cell Signaling Inc.). C. BID is cleaved to jBID when cells were treated with both VELCADE and TRAIL. PC3 cells were treated with VELCADE (100 nM) and TRAIL (50 ng/ml) in the absence or presence of SP600125 for 2 hours, and BID cleavage was examined by immunoblotting using BID antibody.

## 2) Synergistic effect with the combination of TRAIL and conventional chemotherapies in vivo:

Animal experiments using xenograft models are underway, and no conclusion has currently been drawn.

## KEY RESEARCH ACCOMPLISHMENTS:

- We found that most of prostate cancer cells were sensitive to TRAIL treatment while normal prostate epithelial cells were resistant.
- We found that high level of constitutively active pro-survival protein kinase Akt existed in TRAIL-resistant LNCaP cells. Inhibition of PI 3-kinase sensitized LNCaP cells to TRAIL.
- We found that the elevated eNOS activity by Akt phosphorylation may contribute to Akt-mediated TRAIL resistance in LNCaP cells.
- We did a yeast 2-hybrid screening to initially identify possible TRAIL receptors-interacting proteins.
- We found that chemotherapeutic agents (etoposide and VELCADE) can sensitize PC cells to TRAIL-mediated apoptosis. Synergistic effect between VELCADE and TRAIL may be partially dependent on JNK activation and JNK-mediated BID cleavage.

## REPORTABLE OUTCOMES:

One manuscript was accepted for publication.

## CONCLUSIONS:

Our in vitro data suggest that TRAIL is effective to induce most of prostate cancer cells to undergo apoptosis but not toxic to normal prostate epithelial cells. This result lay a solid foundation for further investigation of TRAIL's effect on prostate cancer cells in vivo. Elucidation of TRAIL signaling pathway and its regulation help us to understand the molecular mechanism of TRAIL resistance. Overcome of the anti-apoptotic mechanism, such as Akt's anti-apoptotic activity, in TRAIL-resistant cells by specific inhibitors will be the key for TRAIL-based therapy.

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## eNOS protects prostate cancer cells from TRAIL-induced apoptosis

Xin Tong, Honglin Li\*

*Children's Memorial Institute for Education and Research, The Robert H. Lurie Comprehensive Cancer Center,  
The Feinberg School of Medicine, Northwestern University, 2300 Children's Plaza, P.O. Box 209, Chicago, IL 60614, USA*

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### Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent anti-cancer agent because it induces apoptosis of most tumor cells with little or no effect on normal cells. In this study, we investigated the effect of TRAIL on human prostate normal and cancer cell lines, and found that the prostate cancer cell lines PC-3, ALVA-31, DU 145 and TSU-Pr1 were sensitive to TRAIL-induced apoptosis, while normal PREC cells and cancer cell line LNCaP were resistant. No correlation was found between the sensitivity of cells to TRAIL and the expression of TRAIL receptors DR4 and DR5, and pro-apoptotic proteins Bax and Bak. However, LNCaP cells displayed a high Akt activity. Furthermore, we found that endothelial nitric oxide synthase (eNOS), one of the Akt substrates, was highly expressed in LNCaP but not in other cells. Inhibition of eNOS activity by NOS inhibitor sensitized LNCaP cells to TRAIL. Moreover, PC-3 cell clones stably expressing eNOS were resistant to TRAIL-induced apoptosis. Taken together, these results indicate that eNOS can regulate the sensitivity of prostate cancer cells to TRAIL, and down-regulation of eNOS activity may sensitize prostate cancer cells to TRAIL-based therapy.

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**Keywords:** Prostate cancer cells; Apoptosis; Tumor necrosis factor-related apoptosis-inducing ligand; Akt; Endothelial nitric oxide synthase

### 1. Introduction

Prostate cancer is the most commonly diagnosed cancer and one leading cause of male cancer death [1]. Unfortunately, there are limited treatment options available for this disease, in addition to androgen ablation [2]. However, metastatic cancers frequently develop from a small number of androgen-independent cancer cells after surgery. Those cells are highly resistant to traditional chemotherapies so that the disease is incurable in later

stages. Therefore, more effective agents should be developed to combat metastatic prostate cancers.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent apoptosis-inducing cytokine that together with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), Fas ligand and Apo3L, constitutes a family of ligands that induce apoptosis through binding their corresponding death receptors [3,4]. Unlike TNF- $\alpha$  and Fas ligand, whose use for anti-cancer therapy has been hampered by their severe toxicity [5,6], TRAIL appears to specifically kill a wide variety of cancer cells in culture and xenografted tumors while leaving most of normal cells and tissues unharmed [7]. Although, it was reported that

\* Corresponding author. Tel.: +1-773-755-6359; fax: +1-773-755-6344.

E-mail address: [h-li2@northwestern.edu](mailto:h-li2@northwestern.edu) (H. Li).

TRAIL induces damage in human hepatocytes [8], preclinical experiments in rodents and nonhuman primates have shown that administration of TRAIL suppresses tumor growth without apparent systematic cytotoxicity [7,9]. Therefore, TRAIL represents a potential tumor-specific anti-cancer agent. Recent studies indicate that the signaling pathway of TRAIL-induced apoptosis is largely similar to the Fas signaling pathway [10]. Five receptors that interact with TRAIL have been identified, including the apoptosis-inducing receptors DR4/TRAIL-R1 and DR5/TRAIL-R2/Killer/ TRICK2 [11–13], the decoy receptors DcR1/TRAIL-R3/TRID (without an intracellular domain) and DcR2/ TRAIL-R4 (with a truncated death domain) [11, 13–15], and osteoprotegerin [16]. Through recruiting adapter protein FADD and caspase-8, engagement of TRAIL to its receptors (DR4 and DR5) triggers the formation of so-called death-inducing signaling complex (DISC), which results in activation of caspase-8 and apoptosis of target cells [10]. Nonetheless, the molecular mechanism underlying the tumor cell-specific cytotoxicity of TRAIL remains largely unclear.

The phosphoinositide 3-kinase (PI3K)-Akt pathway is a key regulator of cell survival through multiple downstream targets [17]. It has been shown that Akt phosphorylates and inactivates multiple pro-apoptotic factors such as BAD [18], caspase-9 [19] and the Forkhead transcriptional factors [20,21], the latter are known to mediate apoptosis by activating the transcription of *FasL* and *Bim* [22]. Previous studies have shown that there is an elevated Akt activity in TRAIL-resistant prostate cancer cells, and down-regulation of constitutively active Akt by PI3K inhibitors reverses cellular resistance to TRAIL [23,24]. Furthermore, overexpression of constitutively active Akt in cells with low Akt activity inhibits TRAIL-induced apoptosis [24]. However, not all prostate cancer cells with low Akt activity can gain the ability to resist TRAIL-induced apoptosis when they express constitutively active Akt [23]. This observation indicates that the protective effect of Akt may be cell type-specific, or other factors may contribute to Akt-mediated resistance to TRAIL-induced apoptosis. In the present study, we examined the cytotoxic effect of TRAIL on human prostate normal epithelial cells (PrEC) and cancer cell lines, and found that endothelial nitric oxide synthase (eNOS), a substrate of Akt, was highly expressed in TRAIL-resistant LNCaP cells. Inhibition of eNOS sensitized

LNCaP cells to TRAIL, while over-expression of eNOS enabled TRAIL-sensitive PC-3 cells partially resistant to TRAIL, suggesting that eNOS activity may contribute to regulation of TRAIL signaling pathway.

## 2. Materials and methods

### 2.1. Reagents

Antibodies were purchased from the following sources: anti-PTEN, anti-Bax (Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-phospho-Akt, anti-Akt (New England Biolabs, Beverly, MA); anti-DR5 (StressGen Biotechnologies Corp, Victoria, Canada); anti-DR4, anti-Bak (Upstate Biotechnology, Lake Placid, NY); anti-eNOS, anti-nNOS, anti-iNOS (Transduction Laboratories, Lexington, KY); anti-Tubulin (Sigma). Anti-caspase-8 and anti-Bid antibodies were a gift from Dr Junying Yuan. L-N<sup>5</sup>-(1-iminethyl)-ornithine (L-NIO) was obtained from Calbiochem (San Diego, CA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst 33342 were from Sigma.

### 2.2. Cell cultures

Human prostate normal PrEC cells were purchased from Bio-Whittaker (Walkersville, MA), and were grown in PrEGM medium (Bio-Whittaker). Human prostate cancer PC-3, DU 145, TSU and LNCaP cells were kindly provided by Dr Z. Wang, and ALVA-31 was provided by Dr S.M. Loop. These cells were grown in RPMI 1640 medium with 10% fetal bovine serum. All cells were maintained on tissue culture dishes at 37 °C with 5% CO<sub>2</sub>.

### 2.3. Expression of recombinant TRAIL

The expression construct was pET-15b-His-TRAIL, which encodes for soluble human TRAIL (residues 95–281). *E. coli* BL21(DE3) transformed with this construct were grown in LB medium to exponential phase, and induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h. Cells were pelleted, resuspended in lysis buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), and sonicated. The supernatant after centrifugation at 12000 × g for

30 min was used for purification of His-tagged TRAIL following the manufacturer's instructions (Novagen).

#### 2.4. MTT assay

$1 \times 10^4$  cells (50  $\mu$ l) were subcultured in RPMI 1640 (phenol red-free) supplemented with 10% fetal bovine serum in a 96-well plate, and treated with or without TRAIL for 24 h at 37 °C and 5% CO<sub>2</sub>. For MTT assays, 5  $\mu$ l of MTT agent (5 mg/ml in phenol red-free RPMI 1640) was added and further incubated for 2 h. Equal volumes of 0.05 M HCl in isopropanol were then added, and cells were disrupted by pipetting up and down. The spectrophotometric absorbance of the sample was measured using an automated 96-well plate reader. The wavelength to measure absorbance was 570 nm, and the reference wavelength was 650 nm.

#### 2.5. Western blotting

Cells were harvested and lysed in sample loading buffer, and cell lysates were subjected to SDS-PAGE followed by transferring to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were blocked in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween-20) containing 5% nonfat dried milk overnight at 4 °C. Membranes were then blotted with various primary antibodies for 2 h at room temperature. After washing three times in TBST, membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature. Proteins were detected by ECL (PerkinElmer Life sciences, Inc., Boston, MA) according to the manufacture's instructions.

#### 2.6. Transfection and selection of stable clone

Plasmids encoding the human eNOS and mutant eNOS (S1179D), a kind gift from Dr Stefanie Dimmeler, were transfected into PC-3 cells by LIPO-FECTAMINE Reagent (Invitrogen, Carlsbad, CA). The control was the vector pcDNA 3.1/Myc-His. At 48 h after transfection, the cells were detached with trypsin and re-plated in the medium with G418 (500  $\mu$ g/ml). After 2-week selection, G418-resistant colonies were expanded in 24-well plate and maintained in medium supplement with G418. The stable cell lines expressing eNOS were screened with anti-Myc antibody.

#### 2.7. Nitrite detection

Quantitative analysis of nitrite was used as an index of NO production. In aqueous solution, NO is rapidly oxidized to two stable breakdown products, nitrate and nitrite, which can be detected by colorimetric means.  $1 \times 10^6$  cells were plated onto a 6-well plate, culture supernatants were collected after 24 h culture and assayed for nitrite using Nitric Oxide Assay Kit (Calbiochem), following the manufacture's instructions. Briefly, samples were aliquoted into 96-well plate. Nitrates were reduced to nitrites by nitrate reductase in the presence of NADH. Total nitrites in the samples and in the standards (0.5–25  $\mu$ M solutions of sodium nitrite) were detected colorimetrically with Griess reagents I and II and measured at 540 nm. Culture medium (in the absence of cells) served as sample blanks.

#### 2.8. Flow cytometric analysis

Cells were trypsinized, washed in PBS and fixed with ice-cold 70% ethanol. Cells were then washed in PBS again and resuspended in PBS containing RNaseA and propidium iodide. A total of 10 000 labeled nuclei were analyzed in a FACScan Flow Cytometer (Becton Dickinson, Mansfield, MA). The numbers of cells with sub-G<sub>1</sub> DNA content were determined as indicators of apoptotic population.

### 3. Results

#### 3.1. Effect of TRAIL on human prostate normal and cancer cells

Recombinant human TRAIL was produced in *E. coli* as a fusion protein containing an N-terminal His-tag, and purified by using standard nickel affinity chromatography. Cytotoxic effects of TRAIL on human PrEC and cancer cell lines (namely, PC-3, TSU-Pr1, DU 145, ALVA-31 and LNCaP) were evaluated by MTT assay. As shown in Fig. 1A, PC-3 and ALVA-31 cells are sensitive to TRAIL, and DU 145 and TSU-Pr1 reveal moderate sensitivity, while LNCaP and normal PrEC cells are fully resistant to TRAIL treatment.

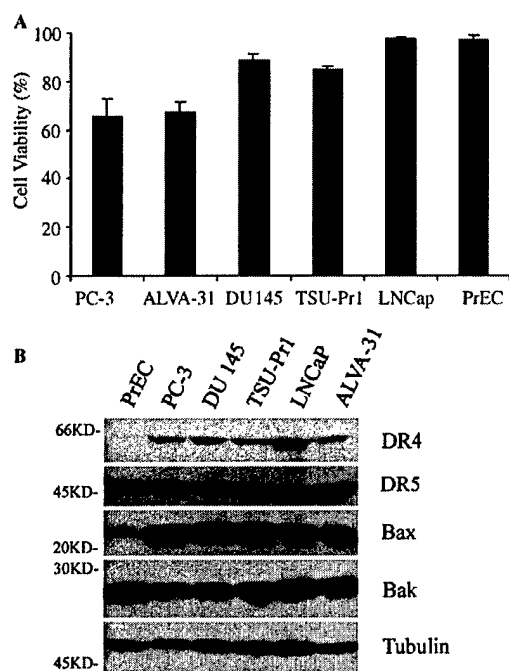


Fig. 1. Effect of TRAIL on cell viability and expression of TRAIL receptors and pro-apoptotic proteins in prostate cancer and normal cells. (A) The effect of TRAIL on cell viability. Cells were treated with TRAIL (500 ng/ml) for 24 h, and viability was measured by MTT assay. Data represent mean  $\pm$  SE. (B) Expression of TRAIL receptors and pro-apoptotic proteins in prostate cancer and normal cells. Cell lysates prepared from prostate normal and cancer cells were probed by immunoblotting with anti-DR4, anti-DR5, anti-Bax, and anti-Bak antibodies.

### 3.2. Expression of TRAIL receptors and pro-apoptotic proteins in prostate cells

To investigate the intracellular mechanisms underlying differential sensitivity of prostate cancer cells to TRAIL, we first examined the expression level of TRAIL receptors (DR4 and DR5). Fig. 1B shows that there is no correlation between the TRAIL sensitivity and the expression of TRAIL receptors DR4 and DR5 among the five prostate cancer cell lines that we tested. On the contrary, higher DR4 expression is found in LNCaP cells. PrEC cells express low level of DR5 but not DR4. Recent study has shown that hepatocytes of Bax/Bak double knockout mice resist death receptor-induced apoptosis *in vivo* [25]. We further examined

the expression of these pro-apoptotic proteins in prostate cancer cells. As shown in Fig. 1B, all cells we tested express comparable amounts of Bax and Bak, indicating that Bax/Bak are not the determinant of TRAIL sensitivity of these prostate cancer cells.

### 3.3. LNCaP cells expresses high levels of active Akt and eNOS protein

Previous studies have demonstrated that LNCaP cells have a high level of constitutive active Akt due to the deletion and mutation at PTEN alleles [24]. Inhibition of PI3K activity using wortmannin and LY-294002 suppresses constitutive Akt activity and sensitizes LNCaP cells to TRAIL [23,24]. As shown in Fig. 2, we found that there is no PTEN expression and the highest Akt activity is observed in LNCaP cells, although total Akt protein levels among these prostate cancer cells are similar. However, DU 145, a TRAIL-sensitive prostate cancer cell line with low Akt activity, is still sensitive to TRAIL even in the presence of constitutively active Akt [23]. This result suggests that there may be some other molecules rather than Akt alone contribute to TRAIL-resistance. Besides BAD, caspase-9 and Forkhead transcription factors, which have been found to be

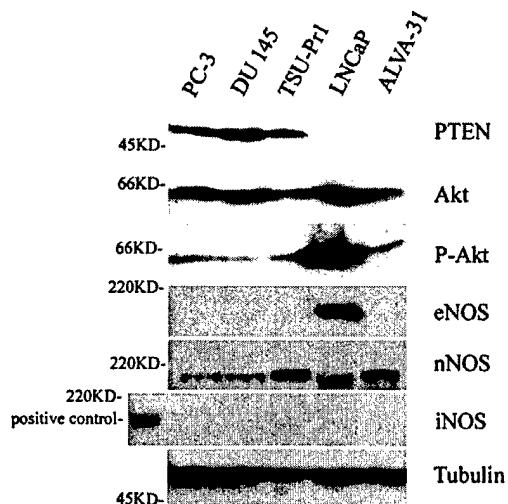


Fig. 2. LNCaP cells express high level of eNOS. Cell lysates from five prostate cancer cell lines were probed by immunoblotting with anti-PTEN, anti-Akt, anti-phospho-Akt (active Akt), anti-nNOS, anti-iNOS and anti-eNOS.



involved in regulation of apoptotic signaling pathway [18–22], eNOS is a recently identified Akt substrate [26, 27]. To explore whether NOS may be involved in TRAIL-resistance, we examined the expression of three isoforms of NOS among the five prostate cancer cells. The data in Fig. 2 shows that only LNCaP cells express eNOS, whereas all of them express nNOS, no one expresses iNOS.

#### 3.4. NOS inhibitor sensitizes LNCaP cells to TRAIL-induced apoptosis

To further examine whether the high-level expression of eNOS in LNCaP cells contributes to TRAIL-resistance, we used eNOS inhibitor to test if inhibition of eNOS can sensitize LNCaP cells to TRAIL. L-NIO is a potent inhibitor of eNOS compared to other arginine analogs [28]. LNCaP cells were pre-treated with L-NIO for 2 h before exposure to TRAIL (500 ng/ml), and cell death was evaluated by nuclear morphology and measured by MTT assay after 24 h treatment (protein synthesis inhibitor cycloheximide was also used as a positive control). As shown in Fig. 3A and B, TRAIL or L-NIO alone does not induce significant cell death (only 2% dead cells), but pre-treatment with L-NIO or cycloheximide sensitizes LNCaP cells to TRAIL-induced apoptosis (38 and 76% cell death, respectively).

#### 3.5. eNOS expression partially inhibits TRAIL-induced apoptosis of PC-3 cells

The potent effect of eNOS inhibitor L-NIO on sensitivity of LNCaP cells to TRAIL treatment suggests that eNOS may be involved in the inhibition of TRAIL signaling pathway in LNCaP cells. To further test this possibility, we used PC-3 cells as a model system to test the effect of eNOS on TRAIL-induced apoptosis, because PC-3 cells are most sensitive to TRAIL among the five prostate cancer cells we tested (Fig. 1A) and have no expression of eNOS (Fig. 2). Wild-type eNOS and mutant eNOS (S1177D) (Mutation of serine 1177 into aspartic acid that substitutes for the negative charge afforded by the addition of phosphate and mimics the activation state induced by Akt) were introduced into PC-3 cells by gene transfection, and stable clones were selected. We randomly selected 30 G418-resistant clones, among them six expressed wild-type eNOS and four expressed eNOS (S1177D), respectively (Fig. 4A).

The clones with high-level expression of eNOS were tested for their TRAIL sensitivity. Empty vector was also introduced into PC-3 cells and G418-resistant clones were selected as a control. We also evaluated the enzymatic activities of eNOS or eNOS (S1177D) in these stable clones by measuring NO level. As shown in Fig. 4B, in comparison to parental PC-3 cells and empty vector clones that produced 6 and 6.6  $\mu$ M of NO, respectively, eNOS clone #8 produced higher level of

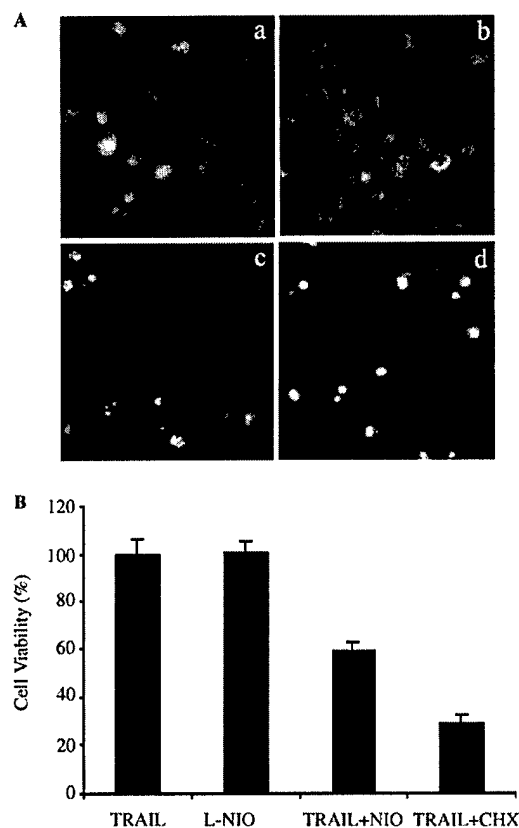


Fig. 3. Inhibitor of eNOS sensitizes LNCaP cells to TRAIL. (A) DNA staining of LNCaP cells treated with 500 ng/ml TRAIL alone (a), 20 mM L-NIO alone (b), TRAIL and L-NIO combination (c) or TRAIL and 10  $\mu$ g/ml cycloheximide combination (d). The cells were stained with Hoechst 33342 (5  $\mu$ g/ml) and nuclear morphology was observed by fluorescent microscope. (B) MTT assay of cell viability. Cell viability of LNCaP cells treated for 24 h with 500 ng/ml TRAIL, 20 mM L-NIO, 10  $\mu$ g/ml cycloheximide alone or in combinations. The viability was measured by MTT assay. Data represent means  $\pm$  SE.

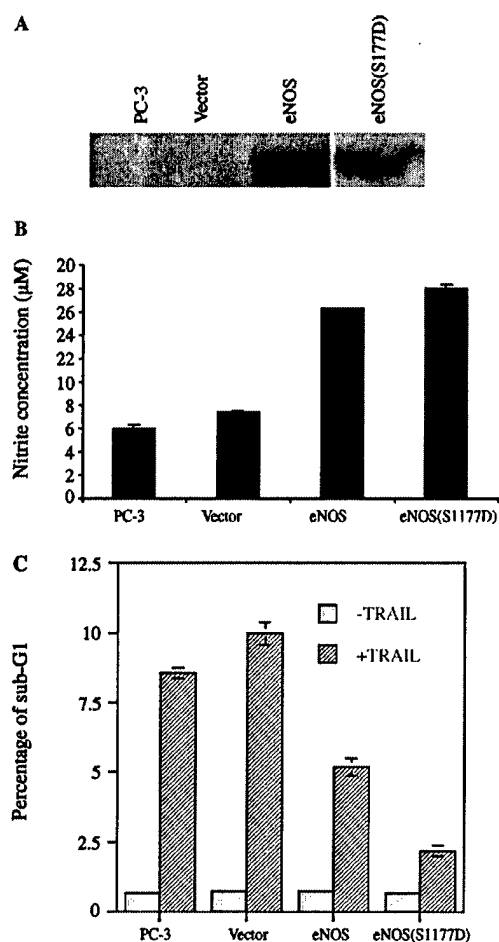


Fig. 4. Overexpression of eNOS in PC-3 cells partially inhibits TRAIL-induced apoptosis. (A) eNOS or eNOS (S1177D)-expressing clones. G418-resistant clones were expanded and cell lysates were probed with anti-eNOS antibody. (B) eNOS activity in eNOS stable cell lines. Culture media from control PC-3 cells, empty vector clones, eNOS and eNOS (S1177D)-expressing clones were collected, and nitrites release was measured by using NO Assay kit. (C) The percentage of cell death evaluated by flowcytometry. Cells ( $1 \times 10^6$ ) were treated with or without TRAIL (500 ng/ml) for 24 h, stained with propidium iodide, and DNA content was analyzed by flowcytometry. The percentage of sub-G1 represented the percentage of cell death.

NO ( $16.2 \mu\text{M}$ ), while eNOS (S1177D) clone #14 produced the highest level of NO ( $17.2 \mu\text{M}$ ).

We further examined whether these eNOS stable cell lines are resistant to TRAIL-induced apoptosis.

Cells were treated with TRAIL (500 ng/ml) for up to 24 h, and the DNA content was analyzed by flow cytometry. The sub-G<sub>1</sub> DNA peak, characteristic of apoptotic cell death, was clearly detectable in PC-3 cells ( $8.6 \pm 0.3\%$ ) or empty vector clones ( $10.0 \pm 0.7\%$ ) treated with TRAIL with respect to untreated controls, while eNOS-expressing cells have much lower sub-G<sub>1</sub> peak ( $5.2 \pm 0.3\%$ ), and the lowest sub-G<sub>1</sub> peak was observed in eNOS (S1177D)-expressing cells ( $2.4 \pm 0.1\%$ ; Fig. 4C). These results demonstrate that overexpression of eNOS in TRAIL-sensitive PC3 cells can inhibit TRAIL-induced apoptosis, further indicating the role of eNOS in regulation of TRAIL signaling.

### 3.6. eNOS expression does not block caspase-8 activation and Bid cleavage

In LNCaP cells, activation of caspase-8 is induced by TRAIL alone, but cleavage of caspase-8 substrate Bid is not detected unless TRAIL is administered in combination with PI3K inhibitor [23,24]. This finding indicates that TRAIL-induced apoptotic signaling in LNCaP cells is blocked at the level of Bid cleavage. In TRAIL-sensitive PC-3 cells, we found both caspase-8 activation and Bid cleavage could be detected by TRAIL treatment alone (data not shown). Since eNOS expression can protect PC-3 cells from TRAIL-induced apoptosis, we further examined whether overexpression of eNOS can also inhibit caspase-8 activation and Bid cleavage. As shown in Fig. 5,

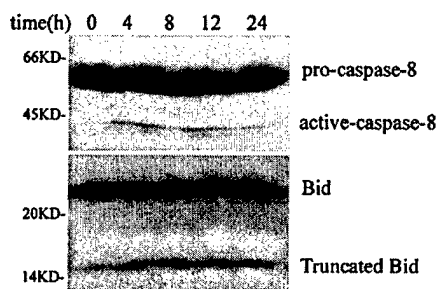


Fig. 5. Overexpression of eNOS in PC-3 cells does not block caspase-8 activation and Bid cleavage. eNOS (S1177D)-expressing PC-3 cells were treated with TRAIL (200 ng/ml) from 4 to 24 h, and cell lysates were probed with anti-caspase-8 and anti-Bid antibodies.

caspace-8 activation and Bid cleavage are still detected in eNOS (S1177D)-expressing PC-3 cells.

#### 4. Discussion

The regulation of TRAIL-induced apoptosis was suggested to be mostly controlled by the expression of TRAIL receptors when the decoy receptors of TRAIL were identified [13]. However, the expression of DR4 or DR5 is necessary to induce the death signal of TRAIL, the expression of DcR1 and DcR2 was not correlated with the sensitivity of TRAIL-induced apoptosis [29], suggesting that the expression level of TRAIL receptors alone is not sufficient to account for the sensitivity of TRAIL-induced apoptosis. On the other hand, the expression of intracellular inhibitors has been proposed to be an essential determinant of cell sensitivity to TRAIL treatment and different cancer cells acquire resistance to TRAIL in different ways. The c-FLIP and PEA-15 proteins were reported to inhibit TRAIL-mediated apoptosis of glioma cells [30], breast and ovarian cancer cell lines overexpressing erbB-2 receptor were found to be resistant to TRAIL [31]. In the present study, we assessed the correlation between expression of eNOS and the sensitivity of prostate cancer cells to TRAIL, which suggests a new mechanism for cancer cells to escape from TRAIL-induced apoptosis.

Initially, we examined the cytotoxic effect of TRAIL on human prostate normal and cancer cells. Cell viability assays demonstrated that prostate cancer cell line LNCaP and normal PrEC cells were resistant to TRAIL. Regulation of TRAIL signaling can occur at multiple points. First, at the ligand-receptor level, the initiation of TRAIL-induced apoptosis may be inhibited by down-expression of TRAIL receptors DR4 or DR5 or over-expression of decoy receptors DcR1 or DcR2 [32]. Although, we found that normal PrEC cells did not express DR4, and had low level of DR5 expression, there was no correlation between the sensitivity of cells to TRAIL and the expression of DR4 and DR5 among the five prostate cancer cell lines that we tested. In contrast, high DR4 expression was found in TRAIL-resistant LNCaP cells. Since an excessive amount of soluble TRAIL was used in our assays, TRAIL decoy receptors should not play an important role in TRAIL sensitivity under present

assay condition. Second, at the level of DISC, the formation of DISC could be inhibited by cFLIP protein that is capable of blocking the activation of caspase-8 [33]. Previous studies [23] and our experiment result (data not shown) indicated that cFLIP does not render TRAIL sensitivity in these prostate cancer cells. Third, in so-called type II cells that the mitochondrial pathway is required to amplify the apoptotic signal [34]. Pro-apoptotic protein Bax/Bak are re-located to mitochondria and required for TRAIL-induced apoptosis [25,35,36]. However, no difference in the expression level of Bax/Bak among these prostate cancer cells was observed. Finally, IAP family proteins may inhibit downstream caspases, which also determine the sensitivity of TRAIL treatment [37]. Still we could not find the difference of the expression of XIAP among these prostate cancer cells (data not shown).

So what determines the resistance of LNCaP cells to TRAIL treatment? It has been reported that a high level of constitutively active Akt activity in LNCaP cells may contribute to TRAIL-resistance [23,24]. Along this line, our present study further identified eNOS, a substrate of Akt, which can regulate the sensitivity of prostate cancer cells to TRAIL-mediated apoptosis. We found that only TRAIL-resistant LNCaP cells expressed high level of eNOS, while no eNOS expression was detected in TRAIL-sensitive cells. eNOS inhibitor L-NIO sensitized LNCaP cells to TRAIL. Finally, the PC-3 cell clones stably expressing eNOS inhibited apoptosis induced by TRAIL, while the parent PC-3 cells were sensitive to TRAIL. Taken together, these findings demonstrate that eNOS plays an important regulatory role in TRAIL signaling pathway in prostate cancer cells.

The role of nitric acid in regulation of apoptosis has been controversial [38]. Elevated level of nitric oxide (NO) can promote apoptosis of some types of cells. As an example, sodium nitroprusside (a NO donor) enhances TRAIL-induced apoptosis via mitochondria-dependent pathway in human colorectal carcinoma CX-1 cells [39]. Long-lasting production of NO may modulate multiple signaling pathways, inducing mitochondrial cytochrome C release [40], up-regulation of p53 expression [41], activation of JNK/SAPK and p38 kinases and altering the expression of Bcl-2 family proteins [42]. Meanwhile, as demonstrated by our study and other

previous studies, NO can also protect cells from apoptosis induced by multiple stimuli such as TNF $\alpha$ , Fas, removal of growth factors or UV irradiation [38]. The anti-apoptotic effect of NO can be achieved through regulating expression of apoptosis-protective genes as heat shock protein 70, Bcl-2, and inhibiting Bcl-2 cleavage, cytochrome *c* release, and ceramide formation as well as directly inhibiting caspase-3, caspase-8 by S-nitrosylation [43–46]. In the present study, caspase-8 activation and Bid cleavage could still be detected in PC3 cells overexpressing eNOS when treated with TRAIL, suggesting that S-nitrosylation of caspase-8 may not be involved, and the mechanisms by which eNOS protects prostate cancer cells from TRAIL-induced apoptosis need be further investigated.

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